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African Journal of
Biotechnology

13 February 2019
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

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Vol. 18(7), pp. 136-143, 13 February, 2019
DOI: 10.5897/AJB2018.16726
Article Number: 7B8F6A660190
ISSN: 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Regulation of a dominant glutathione *S*-transferase and Na^+/K^+ by spermidine under salinity in onion

Tanjina Islam[1,2], Md. Rezwan Molla[1], Reshma Sultana[3], Md. Bazlur Rahman[4], Munshi Rashid Ahmad[5] and Md. Motiar Rohman[1*]

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Received 6 December, 2018; Accepted 29 January, 2019

Spermidine (Spd) is one of the most important polyamines (PAs) showing important roles in growth, development and stress responses in plants. The role of Spd in regulation of a dominant glutathione *S*-transferase (GST, E.C.2.5.1.18) and ionic balance of Na^+ and K^+ in onion leaves were examined. Onion GSTs were separated from fresh bulb tissue using DEAE-cellulose chromatography. Three GSTs were eluted at 56, 120 and 169 mM of KCl. Among them, GST2 containing >60% of total activity was termed as dominant GST and it was subsequently purified using affinity chromatography *S*-hexylglutathione-agarose. This purified protein was used for western blotting analysis of the GST in leaves of two months onion seedlings imposed on NaCl induced 16 dSm^{-1} salinity with or without foliar spray of 100 µM Spd on 1st, 3rd, 5th and 7th days of stress. The results suggested that dominant GST accumulated by salinity in onion seedling with or without Spd spray. However, at early saline stress, accumulation was significantly stronger in leaves without Spd than in those with Spd. At 7th day, GST band was increased and apparently seemed to be intensified in Spd sprayed leaves than those without spray. The values of Na^+/K^+ suggested that Spd maintained ionic balance better at early stage of stress than late stage.

Key words: Onion glutathione *S*-transferases (GST), ionic balance, salt stress, spermidine.

INTRODUCTION

Glutathione *S*-transferases (GSTs, E.C.2.5.1.18) are a multigene family of isozymes, known to catalyze conjugation of tripeptide glutathione (GSH) to wide variety of electrophilic and hydrophobic substrates. GSTs

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are a famous super family of enzymes for their role in detoxification reactions. It is well established that GSTs conjugate GSH to a variety of electrophilic compounds of both exogenous and endogenous origins (Cummins et al., 2011). GSTs have been found in all the organisms including bacteria and fungi (Frova, 2006; Perperopoulou et al., 2017). Although, primarily, plant GSTs were comprehensively studied for herbicide detoxification; recently, specific members of this family have been reported to confer tolerance toward herbicide in many species (Chronopoulou et al., 2017). GSTs catalyze the conjugation of thiol group of GSH and electrophilic substrate. The conjugate is either sequestered into the vacuoles or transferred from the cells by ATP-dependent transporter. In addition to herbicide detoxification, GSTs are involved in hormone biosynthesis, tyrosine degradation, breakdown peroxide breakdown (Oakley, 2011), stress signaling proteins (Loyall et al., 2000), nodule formation (Dalton et al., 2009) as well as ligandins for flavonoid-binding proteins (Mueller et al., 2000). GSTs have also been reported in involvement of different biological processes such as modulation of cell signaling kinases, ion channels, redox homeostasis and post-translational glutathionylation of proteins (Dixon et al., 2010). Plant GSTs have been reported to increase tolerance in different plant species under abiotic stresses (Ding et al., 2017) including heavy metal (Zhang et al., 2013), ultra-violet (UV) radiations (Liu and Li, 2002), salinity (Rohman et al., 2016a) and drought (Rohman et al., 2016b).

Polyamines (PAs), including diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), are abundant low-molecular-weight aliphatic amines involved in different biological and physiological process of growth and development of plants (Liu et al., 2015) and are also known for their anti-stress and anti-senescence effects due to their acid neutralizing, antioxidant and cell membrane stabilizing activities (Zhao and Yang, 2008). Due to their cationic nature at physiological pH, PAs are known to interact with proteins, nucleic acids, membrane lipids and cell wall constituents resulting in stabilizing these molecules (Khare et al., 2018). Apart from these, PAs are reported to involve in biotic and abiotic stresses defenses (Alcázar et al., 2006). It has been shown that a higher PAs concentration in cell correlates with plant tolerance to a wide array of environmental stresses (Liu et al., 2015). Several biochemical and physiological effects were provoked by exogenously applied PAs including Spd under environmental stress. Exogenous Spd was effective in enhancing the activity of peroxidase under salinity stress and the salt-induced increase in reducing sugar and free proline level was further promoted by Spd in indica rice (Roychoudhury et al., 2011). Moreover, it has been shown that over expression of Spd synthase gene in transgenic *Arabidopsis thaliana* maintained higher levels of Spd content and enhanced tolerance to salinity, chilling, hyperosmosis and drought comparative to the wild-type plants suggesting important role of Spd in stress signaling pathway to enhance stress tolerance mechanism for plants (Kasukabe et al., 2004). Onion is a model crop for GST showing higher GSH dependent detoxification enzymatic activity than other vegetable crops (Rohman et al., 2009). In our previous study, Spd up-regulated glutathione and ascorbic dependent enzymatic antioxidants in onion leaves conferring tolerance to salinity (Islam et al., 2016). In that study, GST activity was induced substantially with or without Spd in salinity stressed onion seedlings. However, specific GST isozyme contributing the activity was known. Moreover, information on ionic regulation by Spd in plant cell under saline condition is still limited. Therefore, the onion GSTs were separated to examine the accumulation of highly expressed GST. At the same time, ionic balance of Na^+ and K^+ by Spd application in onion seedlings under salinity was also reported.

MATERIALS AND METHODS

Seedlings of two months old (*Allium cepa* L. var BARI Piaj-3) were used as plant material. They were grown in plastic bucket (30 L), under green house of Bangladesh Agricultural Research Institute (BARI). Homogenous mixture of organic matter and soil were used as growing media in the buckets and 10 seedlings were allowed to grow. Two months old seedlings were imposed to salinity stress by adding NaCl saline solution (10 gL^{-1}) to increase salinity of 16 dSm^{-1} at 50% field capacity. An EC meter (Hanna 993310) was used to measure salinity level. Spd at 100 µM concentration was sprayed twice daily. Soil surface of the bucket was sealed with rock and polythene to maintain the soil moisture. This condition was maintained for seven days. A control without salinity and Spd was maintained under same condition. Data were measured in fully expanded leaves at 1st, 3rd, 5th and 7th days of stress.

Extraction of soluble protein for Western blotting

Onion leaf tissue (0.5 g) was homogenized in 1 ml of 50 mM ice-cold potassium-phosphate (K-P) buffer (pH 7.0) by mortar and pestle containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. Homogenates were centrifuged at 11,500×*g* for 10 min and the supernatants were used for Western blotting. All procedures were performed below 4°C (Rohman et al., 2009)

Measurement of Na^+/K^+

The sap was extracted from leaves and put on compact Na^+ ion meter (Horiba-731, Japan) and compact K^+ ion meter (Horiba-722, Japan) to estimate the Na^+ and K^+ ions in leaves. The Na^+/K^+ ratio was calculated from the estimated values.

Determination of protein

The protein concentration in the leaf extracts was determined according to the method of Bradford (1976) using BSA as a protein standard.

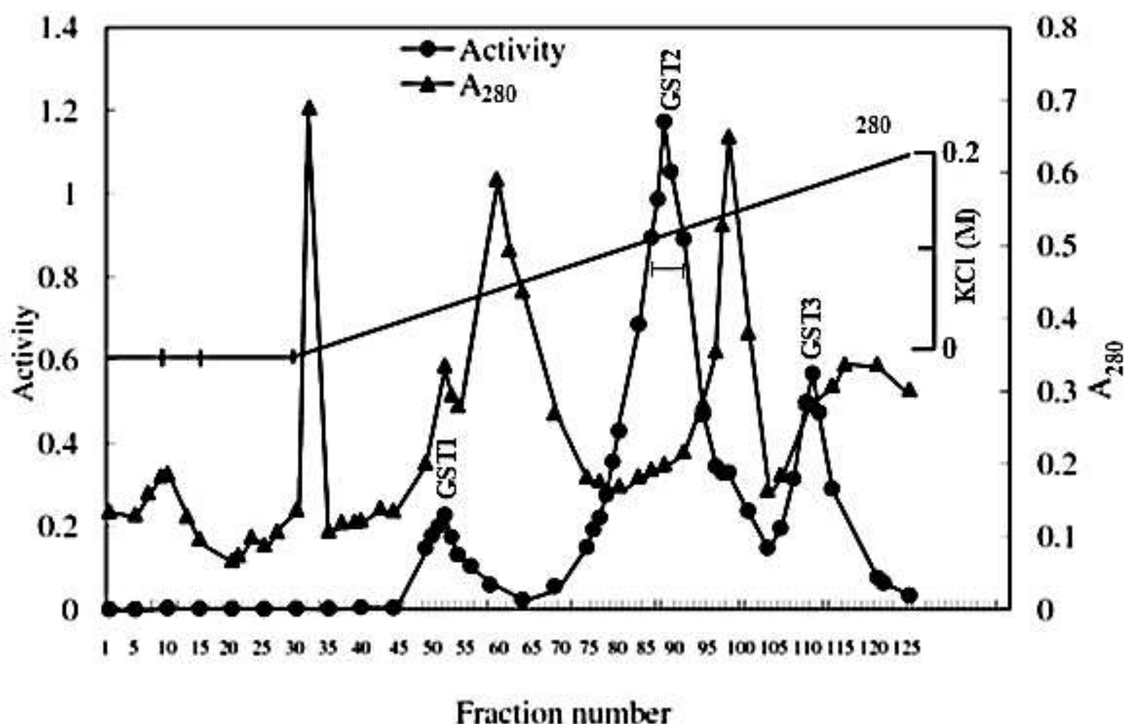

Figure 1. Typical column chromatography of DEAE-cellulose of soluble proteins prepared from 150 g onion seedlings. For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (♦) were determined. GST activity was expressed as μmol min^{-1} ml^{-1}. The curve showed the gradient solution of KCl (0-0.2 mM). Fractions of GST2 above the bar were pooled for further purification.

Separation and purification of GST and production of polyclonal antibody

For GSTs separation and purification, onion bulb tissue was used as onion bulb showing higher GST activity than leaf (Rohman et al., 2010). For separation of GSTs, 150 g fresh onion bulb tissue was homogenized in an equal volume of 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1% (w/v) ascorbate and 10% (w/v) glycerol and the supernatant was precipitated with ammonium sulfate at 65% saturation. GSTs were separated by anion exchange column (1.77 cm i.d.×20 cm) of DEAE-cellulose (DE-52; Whatman, UK). The fractions corresponding to the high GST active peaks were combined as the GST pool which was further purified by *S*-hexylglutathione-agarose (Sigma, St. Louis, MO) eluting with 1.2 mM *S*-hexylglutathione. Purified protein was used to prepare polyclonal antibody.

Production of polyclonal antibodies against GST

A rabbit (weighing about 2.5 kg) received subcutaneous injections of a 0.5 mg of purified GST protein in Freund's complete adjuvant at several sites. After two weeks, the rabbit was given a first booster injection of 0.5 mg of the purified GST protein in incomplete adjuvant, and then a second booster injection of 0.5 mg of the purified protein in incomplete adjuvant was given two weeks after the first booster injection. Blood was taken from the ear vein one week after the second booster injection. The blood serum was used as a polyclonal antibody (Rohman et al., 2009).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE was done in 12.5% (w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970). The western blot was done following the protocol of Perkin-Elmer Life Science Inc.

Statistical analysis

Data of Na^+/K^+ was analyzed by Statistix 10 statistical program following complete randomized design (CRD) and the mean differences were compared by Tukey's tests. P values<0.05 were considered to be significant.

RESULTS AND DISCUSSION

In application of soluble extract of onion bulb on a DEAE-cellulose chromatography, a total of 125 fractions, each containing 5 ml elution, were obtained (Figure 1). GTS activity toward CDNB and absorbance at 280 nm (A_{280}) were measured and plotted as shown in Figure 1. These three GSTs were eluted through the anion exchange chromatography and were named as GST1, GST2 and GST3 (Figure 1).

Table 1. Elution pattern of onion GSTs from DEAE-cellulose chromatography.

GST	Activity (%)	Total protein (mg)	Elution point (mM of KCl)
GST1	6.48	4.35	56
GST2	63.38	6.02	120
GST3	30.14	4.88	169

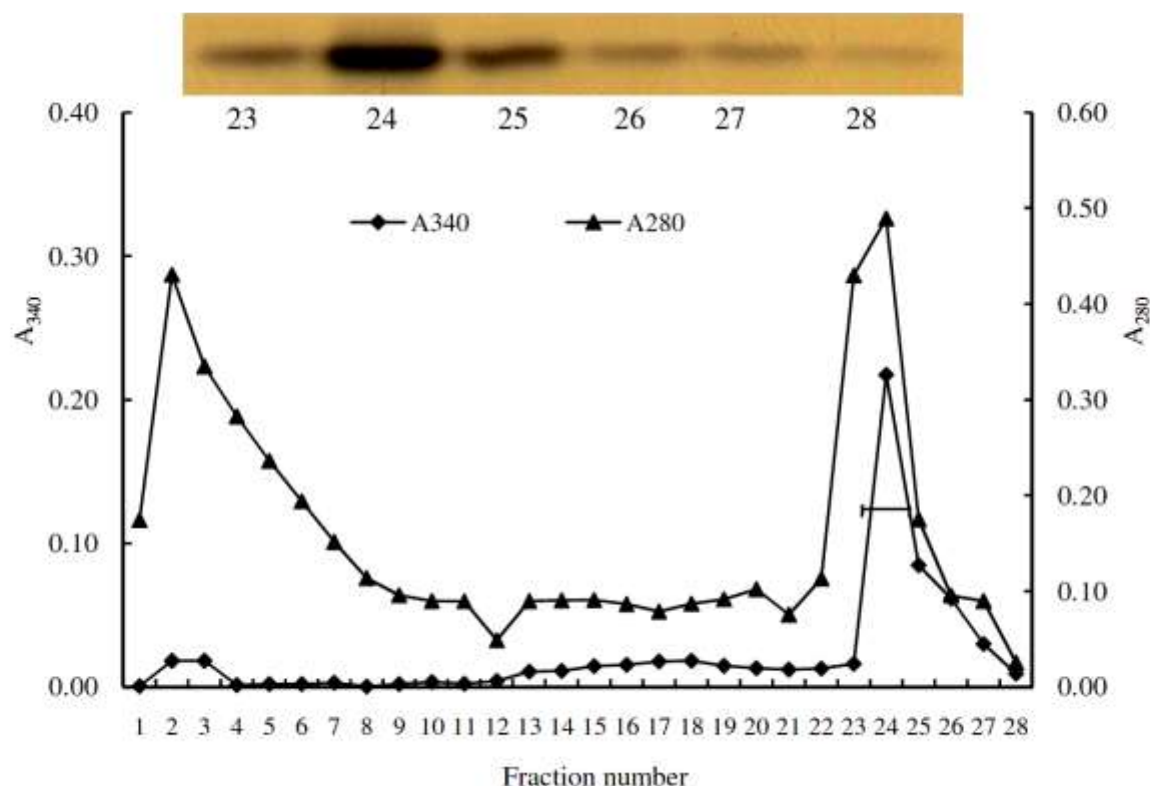

Figure 2. Typical affinity chromatography of *S*-hexylglutathione-agarose. For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (♦) were determined. Activity A_{340} changes for 1 min and absorbance A_{280} were measured.

The three GSTs were eluted at 56, 120 and 169 mM of KCl, respectively, while GST1, GST2 and GST3 contained 6.48, 63.38 and 30.14% of total activity, respectively (Table 1). As GST2 contained 63.38% of the total activity, it was termed as dominant GST, and it was further subjected to purification by affinity chromatography of *S*-hexyl glutathione-agarose.

The high active GST peaks were pooled and pass through the affinity chromatography. The GST was eluted with 1.2 mM *S*-hexyl glutathione. Total 28 fractions, each containing 2.5 ml, were collected. Among them, fractions 21 to 28 are *S*-hexyl glutathione eluted fractions (Figure 2). The GST activity and absorbance (A_{280}) of the fractions showed that *S*-hexyl glutathione fractions are eluted with high GST activity.

The purity of highly active fractions (fraction 23, 24, 25, 26, 27 and 28) were tested by SDS-PAGE following silver staining (Figure 2). The silver staining of the active fraction revealed that the fraction 24 contained highly purified GST with intensified band. However, in this study, fractions 24 and 25 were pooled. The summary of the purification is shown in Table 2. The purified GST had specific activity of 16407.6 nmol min^{-1} mg^{-1} protein with recovery percentage and purification fold of 2.81 and 29.5, respectively. The purification process was repeated several times to produce necessary protein required for developing polyclonal antibody in rabbit antiserum.

Western blot analysis of soluble extract of onion leaves is as shown in Figure 3. The protein bands showed significant accumulation of the dominant GST under

Table 2. Summary of purification of GST from onion bulb.

Fraction	Specific activity (nmol min^{-1} mg^{-1} protein)	Total activity (mMol min^{-1})	Total protein (mg)	Recovery yield (%)	Purification fold
Homogenous	556.20	200.9	361.20	100	1
$(NH_4)_2SO_4$ppt	1539.10	162.07	105.30	80.62	2.77
DEAE-cellulose	8193.10	49.33	6.02	24.55	14.73
S-hexyl glutathione-agarose	16407.6	5.65	0.35	2.81	29.5

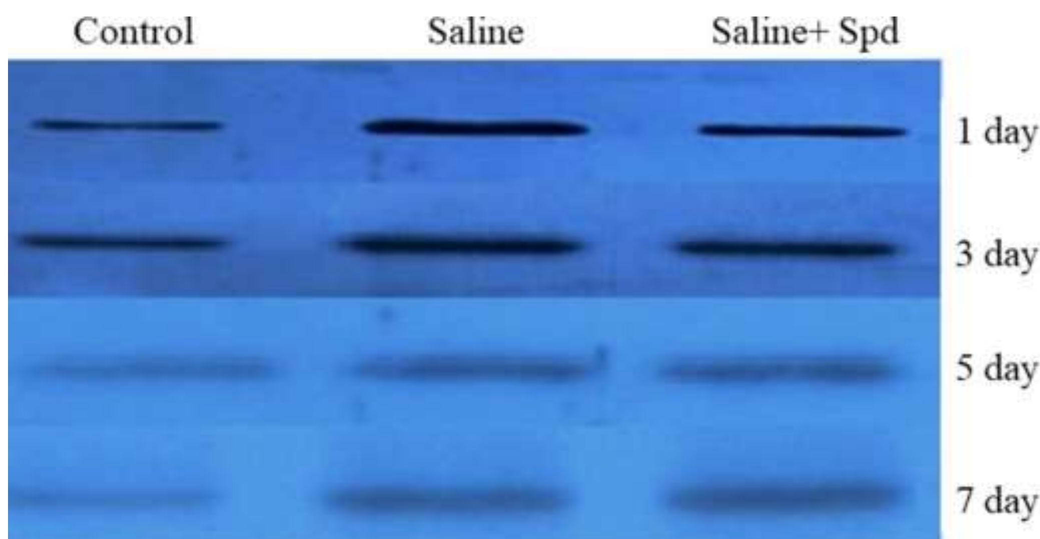

Figure 3. Western blot analysis showing accumulation of onion GST under salinity with the application of exogenous Spd (In each lane, 70 µg protein was applied).

salinity stress in the presence and absence of Spd (Figure 3).

The intensification of bands in Western blot clearly indicated the accumulation of the GST in onion leaf under salinity with or without Spd application (Figure 3). However, concentration of the GST was lower in the leaves with Spd than those without Spd. However, accumulation of the GST increased with stress duration, particularly in Spd sprayed leaves. Significant induction of GST could have significant biological role in stress mitigation onion seedlings under salinity. GSTs are an ancient and diverse group of multi-functional proteins that are widely distributed amongst living organisms. Early plant GST research is focused on the role of GSTs in herbicide resistance and vacuolar sequestration of anthocyanins (Edwards and Dixon, 2000). On one hand, the substantial accumulation of the GST under salinity might play important physiological role like vacuolar sequestration of flavonoids like quercetin (Fini et al., 2011) as quercetin is the most abundant physiological substrate in onion bulb (Rohman et al., 2009). On the other hand, high activity might be associated with recycling and stabilizing flavonoid (Dixon et al., 2011). GSTs have also been shown to possess GST activity towards 4-hydroxy-2-nonenal (HNE) (Gronwald and Plaisance, 1998), a naturally occurring lipid peroxidation product that causes oxidation and alkylation of proteins and DNA. In addition, GST activity allows GSTs to detoxify electrophilic compounds by catalyzing their conjugation to GSH, while GSH peroxidase (GPX) activity allows GSTs to directly detoxify lipid and DNA peroxidation products (Marrs, 1996). Therefore, it is also possible that the induced accumulation of GST in the present study as well as induced GST activity in our previous study (Islam et al., 2016) could detoxify HNE as well as MDA, another natural lipid peroxidation product, under saline stress condition. Moreover, GST may be involved in program cell death like leaf senescence (Kunieda et al., 2005). Therefore, the induced GST in the presence and absence of Spd can help in leaf senescence in onion under salinity stress. However, comparatively lower accumulation of GST in the presence of Spd is not clear. One of the possibilities may be that lessened stress of onion plants in the presence of

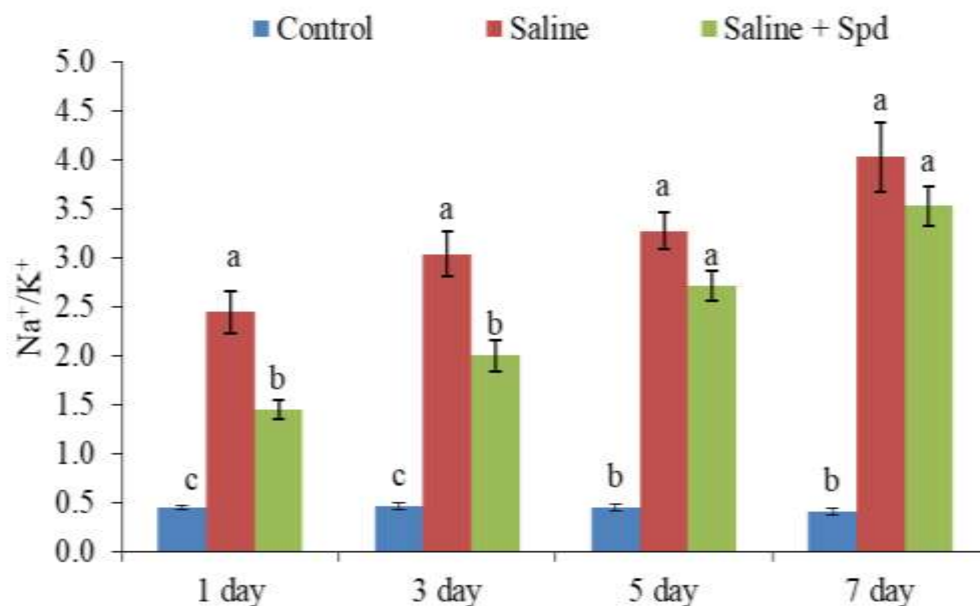

Figure 4. Effect of exogenous Spd on Na^+/K^+ in leaves of onion seedlings under saline stress. The similar letters on the bar within a specific day are not significant at P≤0.05.

Spd can decrease the synthesis of the enzyme. In our previous study, it was suggested that Spd could be useful for saline stress up to 3 days of stress, and in prolonged stress, bursts of ROS and MDA were found even in the presence of Spd (Islam et al., 2016; Rohman et al., 2017). Therefore, higher accumulation of GST at later stage could also be related to oxidative stress.

The value of Na^+/K^+ increased sharply and continuously in onion leaves with stress duration (Figure 4). Data also showed that application of Spd reduced Na^+/K^+ under saline stress while significant reduction was found at the 1st and 3rd days (Figure 4).

It is established that accumulation of Na^+ and loss of K^+ ions occurs under salinity stress. Previously, lower accumulation of Na^+ in the presence of Spd treatment in rice was associated with prevention of electrolyte and amino acid leakage and chlorophyll loss under saline stress (Parvin et al., 2014; Duan et al., 2008). Spd is also reported to interact with phospholipids or other anionic groups of membranes, and thus, it stabilizes the membrane in stress environments (Amri et al., 2011).

Maintenance of cellular Na^+/K^+ is essential for growth and development of plants. Salinity causes the ion toxicity to increase oxidative damage (Gill and Tujeta, 2010). In this study, the ratio of Na^+/K^+ increased under salinity stress conditions and the values decreased in Spd sprayed seedlings (Figure 4). Under salinity, exogenous Spd has been reported to enhance the activities of stress mitigating enzymes like *S*-adenosylmethionine decarboxylase and diamine oxidase (DAO) in zoysia grass cultivars (Li et al., 2016) and reduced the activities of arginine decarboxylase. Exogenous Spd treatment regulates the metabolic status of PAs in tomato (*Solanum lycopersicum*) under salinity-alkalinity stress by providing tolerance (Hu et al., 2012). Spd has also been shown to inhibit the extent of salt-induced protein carbonylation. On the other hand, Spd promoted the synthesis of salt-induced anthocyanin, reducing sugar and proline levels in rice (Roychoudhury et al., 2011). These results suggested the stress tolerant role of SPd under salinity stress. In our previous study, Spd increased the activities of ployamine oxidase (PAO) and DAO along with ROS and MG detoxifying enzymatic and non-enzymatic antioxidants in onion (Islam et al., 2016; Rohman et al., 2017). It also maintained higher chlorophyll contents in onion under salinity stress. In another study, improved tolerance was found with higher PAO and DAO activities as well as antioxidant activities in maize seedlings by exogenous Spd (Akter et al., 2018). In the present study, application of Spd reduced the Na^+/K^+ (Figure 4) in the leaves of onion seedlings. This result suggested that use of Spd reduced Na^+ ion toxicity as well as increase K^+ uptake. Previously, reduced Na^+ content and higher chlorophyll values, higher variable fluorescence/maximum fluorescence values and a higher net photosynthetic rate were reported in Spd applied wheat seedlings under saline stress (Gill and Tuteja, 2010). Similarly, reduced uptake of toxic Na^+ ion concurrently with higher uptake of beneficial ions such as Ca^{2+} and Mg^{2+} by Spd treatment was also reported to

attribute better salt tolerance in plants (Anjum, 2011).

Conclusion

In this study, three GST isozymes were found, and among them, the dominant GST contained 63.38% of the total activity. The specific activity of the purified GST was 16407.6 nmol min^{-1} mg^{-1} protein with recovery and purification fold of 2.81 and 29.5, respectively. The Western blotting analysis suggested the accumulation of the GST under salinity alone at early stage of stress while in the later stage in Spd treated seedlings. The ration of Na^+/K^+ indicated the importance of Spd in improving ionic balance Na^+ and K^+. Thus, these results along with our previous result suggested improved tolerance by Spd spray in onion seedlings under saline stress. However, the lower accumulation of the dominant GST at early stress by Spd thrusted more research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 18(7), pp. 144-154, 13 February, 2019
DOI: 10.5897/AJB2018.16720
Article Number: 02F1AD760200
ISSN: 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Genetic structure of cassava populations (*Manihot esculenta* Crantz) from Angola assessed through (ISSR) markers

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Received 28 November, 2018; Accepted 6 February, 2019

Eighteen ISSR primers (between Simple Repeated Sequences) were used to estimate the genetic diversity of Angolan cassava populations in order to identify genetic variabilities and their potential to be used in the diversification of cassava crops in Angola. In order to do so, three populations from three Angolan provinces were analyzed (Cuanza Norte, Uige and Malanje), which totaled 40 genotypes. In total, 116 bands were amplified with 93.24% polymorphism and 6.4 markers per ISSR primer, on average. Nei's gene diversity (H) and Shannon indices (I) applied to the analyzed accessions presented values ranging from H = 0.20 to 0.24 and I = 0.29 to 0.50 which are intermediate values. The total gene diversity (HT) was 0.3113, and it showed high heterozygosity in *Manihot* populations; this outcome indicated that these populations presented gene variability. ANOVA showed 10.41% total variance between populations and 89.59% within them. Molecular featuring revealed gene diversity in each of the assessed populations; there was gene structuring between populations.

Key words: Gene diversity, molecular markers, gene flow, polymorphism.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) follows rice and maize in the rank of calories and is the third source of the most important calories in Africa, Asia and Latin America. The species is featured as food safety culture in these regions, since small farmers are responsible for most of its cultivation, mostly in marginal lands (FAO, 2014). Cassava is cultivated in more than 60% of the territory used for agriculture in Angola (Muondo, 2013). It is also part of the Brazilian historical context in food, social, economic and cultural plans. Subsistence cultures

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mitigate starvation among the poorest populations in Brazil, mainly in family farms in the vast Northwestern semiarid region (Afonso et al., 2014).

Cassava can become the main raw material for a series of products manufactured with its shoots and roots, which can increase the demand for this species and help agricultural transformation and economic growth in developing countries (FAO, 2018). It is common to consume young cassava leaves in Africa; this dish is called quizaca in Angola; in Mozambique, it is known as matapa, the most popular Mozambican dish. In Brazil, cassava leaves are consumed in a dish called maniçoba (Afonso et al., 2014), its roots are boiled and fried, or eaten in the form of flour and crumbs, in filling and *pirão* (Oliveira, 2011).

The genetic structure of a species can be defined as the distribution of the genetic variability between and within, populations (Brown, 1978; Loveless and Hamrick, 1984). According to Hamrick (1982), genetic structure refers to the homogeneous distribution of alleles and genotypes in space and time. This process results from the action of evolutionary processes (migration, mutation, selection and genetic drift) that act in species and populations. Cruz et al. (2011) highlight that the knowledge about the genetic structures of a population enables predicting, or even clarifying, the ecological and genetic phenomena acting in it.

Studies on the genetic diversity of populations evidence that markers can be used for different means, such as to evaluate the potential of the genetic resources available, to generate connection maps and to detect quantitative trait loci (QTLs), in its association with agronomic features (Benko-Iseppon et al., 2003), as well as to detect polymorphism between genotypes in order to help plant enhancement programs (Ferreira and Grattapaglia, 2008).

Microsatellite and allozyme markers were the ones, among the many existing genetic markers, presenting mendelian inheritance mechanism and codominant expression. This process allows identifying the heterozygous and homozygous genotypes of individuals and became the main information to estimate different parameters of genetic interest (Ferreira and Grattapaglia, 1998; Grattapaglia, 2001; Souza, 2001). However, the most important difference between the two categories of markers refers to their levels of polymorphism. According to Estoup et al. (1998), microsatellite markers are more polymorphic than isoenzymes in natural populations showing more alleles and overall heterozygosity higher than 0.5.

Knowing the polymorphic information content (PIC) is extremely useful to determine the number and size of families adopted for QTL mapping, PIC is the probability of a parent to be a heterozygous in a *locus* and of the other parent to have a different genotype. Families can be chosen based on parental heterozygosity in order to improve marker information by selecting the most informative markers (Zhu et al., 2001). Accordingly, based on the scarcity of information about studies on the genetic structure of cassava populations in Angola, the aim of the present study was to estimate the genetic diversity of cassava populations in Angola through ISSR molecular markers. It was done in order to identify their genetic variability and potential to be used in cassava cultivation diversification in Angola.

MATERIALS AND METHODS

Leaf samples of 40 genotypes from three *Manihot* populations were collected in the Experimental Station of Malanje Food Company, located at latitude 8° 49' South and longitude 13° 13' West (IGCA, 2016), altitude 368 m and total area 8960 m^2. According to the INAMET classification (2004), the climate in the region is sub-tropical humid with mean annual temperature of 26°C, thermal amplitude of 14°C, relative humidity between 80 and 85%, and mean annual rainfall between 1000 and 1200 mm.

Genotypes collected in each one of the three provinces (States) were the populations taken into account: Cuanza Norte (Ndalatando), Malanje and Uíge (Table 1 and in Figure 1). The collected samples were stored in aluminium paper, labeled and sent to Embrapa Cassava and Fruits Molecular Biology Laboratory in Cruz das Almas City, Bahia State, Brazil. Next, they were stored in freezer at -20°C until DNA extraction.

DNA was extracted through the CTAB method by Doyle and Doyle (1987), with modifications. The protocol encompassed 0.1 M of Trisat pH 8.0; 1.7 M of NaCl at 5 M; 20 m Mof EDTA at 0.5 M; 2.4% CTAB; 2.0% (p/v) PVP-40 and 0.4% (v/v) of pre-heated β-mercaptoethanol at 65°C in water bath. The DNA stock was stored in freezer at -20°C after its extraction. Total DNA concentration was estimated in 1% agarose gel stained in Blue Juice. Next, DNA was diluted and stored in gelatin until its use. Polymerase Chain Reaction (PCR) was conducted with 32 Embrapa ISSR primers. The PCR mix (Promega Kit) was composed of 2.5 mM $MgCl_2$ (25 mM); 200 mM dNTP (2.5 mM); 1.0 X Go Taq 5X; primer (0.4 µM); 2.0 U Taq (1 U/ 0.2 µl); DNA (15 ng) and milli-Q water at final volume 15 µL per sample. PCR reactions were generated in automatic thermocycler Applied Biosystems with 96-well blocks, when samples were initially denatured at 94°C for 3 min. This process was followed by 39 amplification cycles, each sample was subjected to 94°C cycles for 40 s, to 72°C for 1 min and 72°C for 5 min. The process was over after the cycles at 72°C for 5 min and cooled at 4°C. PCR products were subjected to electrophoresis in horizontal cube, in 3% agarose gel (p/v) buffer and stained in ethidium bromide (1.5 ng/µL) and in reaction buffer 15 ng of DNA, 0.4 primer, 200 mM dNTP 2.5 mM, 1.0 X Go Taq 5X, 2.5 mM $MgCl_2$ 25 mM, 2.0 U Taq (1 U/0.2 µL) and milli-Q water in order to complete the reaction volume at 100 V for 3.5 h; 1Hb molecular weight markers were used (Ladder Invitrogen Norgen). The gels were removed from the cube and photographed under ultraviolet light to reveal DNA fragments stained in Blue Juice after the electrophoresis. Primers presenting the largest number of fragments and good resolution were selected; results of primers that showed low intensity or low definition bands were discarded. A binary matrix was elaborated based on the presence (1) and absence (0) of *loci.*

Initially, the potential of markers to estimate the genetic variability of genotypes was examined by measuring marker information through band counting. Band features of indicators such as total number of bands (TNB), number of polymorphic bands (NPB) and the percentage of polymorphic bands (PPB) were obtained. Three indices were used to determine the most informative ISSR primers: polymorphic information content (PIC), marker index (MI) and resolution power (RP).

Table 1. Number of accessions (N), province (State), county and their respective geographic coordinates.

Province	Number	County	Geographic coordinates
Cuanza Norte	8	Ndalatando	07° 49’ 27.3” S 015° 01’ 24.1 ”W
Malanje	26	Malanje- Cambondo do KuigeColiny	09° 39’ 44.6” S 16° 19’ 46.0 ” W
Uige	6	Kiongua	07° 49’ 02.8” S 015° 03’ 0.31 ” W

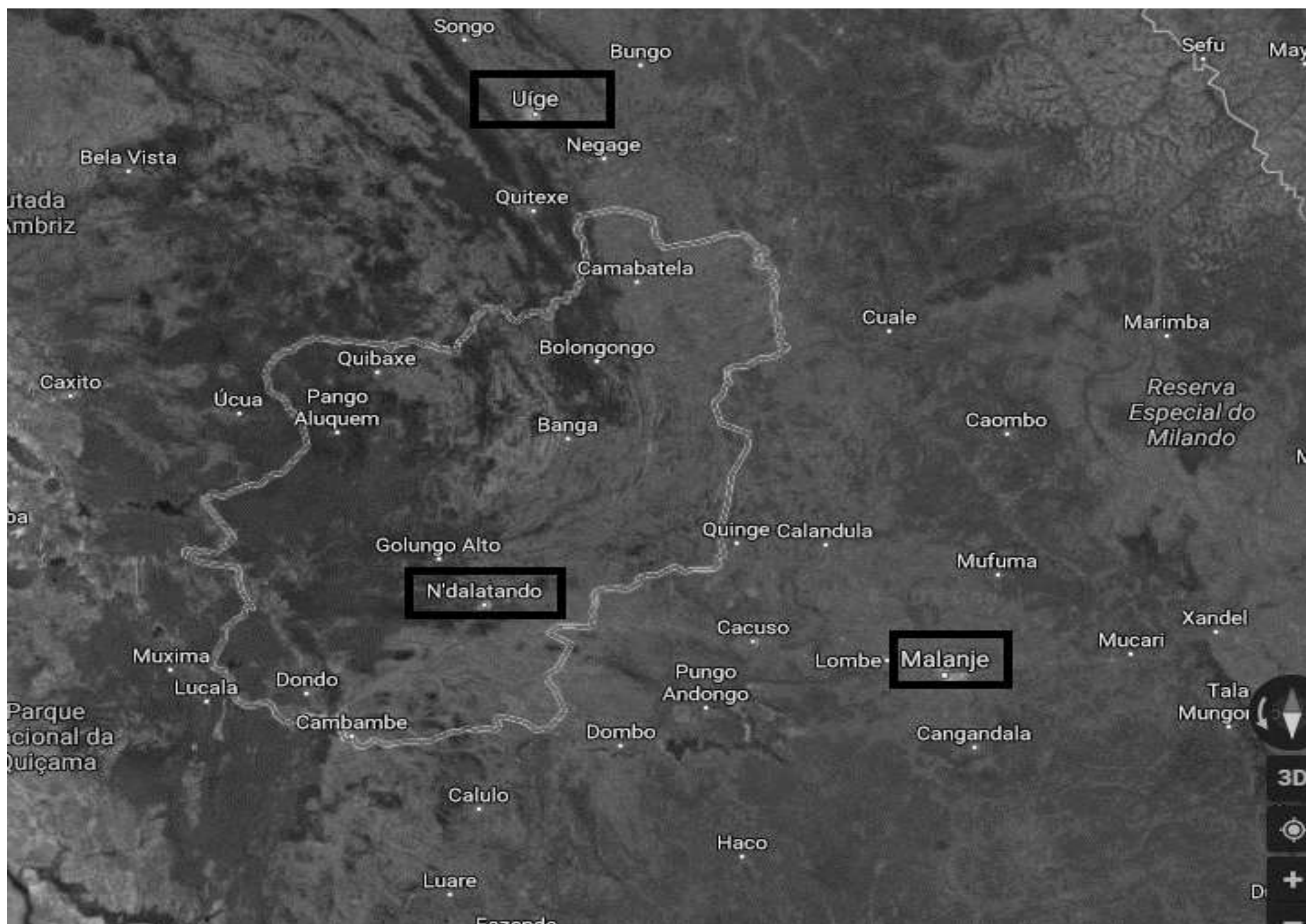

Figure 1. Geographic map showing the collection location of the 40 cassava accessions.

Polymorphic information content (PIC)

PIC values for each ISSR locus were calculated through:

$PICi = 2fi\,(1 - fi)$

Where PICi is the polymorphic information content of primer *i*, f*i* is the sequence of amplified loci (presence of bands) and 1-f*i* is the frequency of null alleles (Roldan-Ruiz et al., 2000).

Marker index (MI)

MI was calculated based on Varshney et al. (2009), wherein:

MI = PIC × EMR

The effective multiple relation (EMR) was calculated as follows:

EMR = n × β,

Where n= total of indicator amplification fragments / total no. of assessed fragments; β = number of polymorphic fragments/total number of amplified fragments.

Resolution power (RP)

RP was calculated according to Prevost and Wilkinson (1999):

$RP = \sum I_b$,

Where, I_b represents the polymorphic loci, I_b can be turned into a

0-1 scale through the following formula:

$I_b = 1 - (2 \times |0.5 - p|)$

Where, *p* is the proportion of genotypes presenting the *locus*.

Firstly, 10 primers presenting higher values for each one of the calculated indices (PIC, MI and RP) were identified to allow the selection of the 10 best ISSR primers for genetic studies focused on cassava varieties. Primers were selected after this identification by using the following criteria: (1) coincident primers among the three best ones for the three indices (PIC, MI and RP); (2) coincident primers among the three best ones for the two indices (MI and RP); (3) primers presenting the highest RP values; (4) primers presenting the highest MI values.

Genetic diversity parameters such as number of effective alleles (Ne), number of observed alleles (Na), genetic diversity of Nei (H) and Shannon index (I) were calculated in the POPGENE software version v.1.32 (Yeh et al., 1997). The analysis of variance was performed in order to check the existing genetic relation between populations and between accessions within each population in the *Manihot* collection. The significance test of the analysis of variance (ANOVA) was conducted with 1000 permutations calculated in the ARLEQUIN software Ver. 3.1 (Excoffier and Lischer, 2005).

The Bayesian grouping analysis was conducted in the STRUCTURE software, v. 2.3.4 (Pritchard et al., 2000) in order to find the number of genetic groups. The tested K number ranged from 1 to 10. The ΔK method by Evanno et al. (2005) was used to find the best K, as implemented in the Structure Havervester (Earl and Vonholdt, 2011).

RESULTS

Thirty-two primers were tested and 18 of them were selected for the genetic diversity study (Table 2), since they were the most informative ones (high polymorphism indices and resolution adequate for the analyses). The selected primers generated 116 bands in total, bands ranged from 4 to 10, thus totaling 6.4 (93.24%) bands per primer, on average; this number indicated increased genetic variability (Table 2). Primers 11 and 27 were the most informative ones and recorded 100 and 90% polymorphism, respectively, and the largest number of bands (10). Primers 42 and 46 (with four bands each) showed the lowest number of bands (Table 2).

PIC recorded mean value of 0.44, with variation ranging from 0.08 (primer 44) to 0.81 (primer 45) (Table 2). MI values of the primers used in the present study ranged from 0.00 (primer 36) to 0.41 (primer 27); mean value of 0.12 (Table 2). Primers 26 and 27 recorded the highest MI values, although primer 27 recorded the highest MI value and was not selected in this study based on moderate PIC values. RP values ranged from 1.63 (primer 36) to 6.13 (primer 26). Primers 6, 7, 11, 26, 27, 36, 39, 44, 53 and 79 presented the highest RP values.

The estimated values of genetic diversity indices in *M. esculenta* populations are shown in Table 3. The percentage of polymorphic *loci* (P) was high and recorded 48.15% in the Uíge population, 61.11% in Cuanza Norte and 92.59% in Malanje. The number of alleles (Na) in the assessed populations ranged from 1.48 to 1.92 and the effective number of alleles (Ne) ranged from 1.37 to 1.59. The Shannon index (I) ranged from 0.29 to 0.50 (Table 3). The distribution of genetic variability between and within, populations was calculated based on Nei (1973) (Table 4). The genetic divergence was 0.1690 and it showed that 16.90% of the genetic variability is observed between populations and that 83.10% of the total genetic diversity (HT) calculated through Nei (1973) reached the estimated mean 0.3113. The observed gene flow value in this study reached 2.4585 (Table 4).

The molecular analysis of variance (ANOVA) applied to the three populations confirmed that most of the genetic variation is within populations (89.59%), 10.41% of the genetic variation was between populations (Table 5). There was significant genetic differentiation (P <0.001) between populations. The inter-population genetic divergence value (F_{TS}) observed in the current study (Table 5) was 0.1041. F_{ST} values between 0.05 and 0.15 suggested moderate genetic differentiation between populations (Wright, 1978). The analysis of the main coordinates (PCoA) was calculated based on coefficients of dissimilarity; its result is graphically presented in Figure 2. The two axes presented 51% total variation, the first axis (PCoA1) recorded 28.54% total variation and the second one (PCoA2), 22.46% of it.

Based on Figure 4, when K=1, genotypes from regions Cuanza Norte, Malanje and Uìge were formed by three great ancestor groups, basically: green, blue and red. Materials from Ndalatando County belonged to group green; group blue was formed by genotypes from Kalandula and Quizenga counties and from Cambondo Colony, whereas Quitoque and Kiongua colonies provided the genotypes in group red. The green group was formed by eight genotypes, group blue by 12 genotypes and the red one by five genotypes. When K=2, it was possible observing that the population in Malanje region basically remained in the Blue group, which is composed of eight genotypes. Although the marker is dominant, there is allelic richness when K=3, and there is allele sharing between one and multiple populations. This group was composed of genotypes from the Malanje and Uíge region and it allowed observing the formation of two ancestor groups: blue and red. Materials from Cambondo do kuige, Comuna do Lombe and Santa Maria colonies belonged to group blue, whereas genotype Mpelo from Quitoque colony belonged to group red. However, the Cambondo do kuige colony and the Lombe commune were formed by two genotypes: Gueti (39) and Kapumba (40), Muringa (36) and Kinzela (37), respectively, whereas Santa Maria and Quitoque colonies by one genotype, Ngana Yuculu (35) and Mpelo (38).

DISCUSSION

The descriptive analysis between *M. esculenta* populations was based on the amplification of ISSR markers. The selected primers generated 116 bands and this number

Table 2. Description of 32 ISSR primers used in the present study and their respective parameters in Angolan cassava.

Number of primer	Primer	AP	T (°C)	TNB	NPB	NMB	% P	PIC	EMR	MI	RP
6	DiCA5'CR	✓	55.0	6	6	0	100	0.24	0.17	0.04	5.18
7	DiCA5'CY	✓	55.0	6	6	0	100	0.47	0.22	0.10	4.13
11	DiGA3'C	✓	46.8	10	10	0	100	0.59	0.46	0.02	5.45
26	DiGT5'CR	✓	54.3	9	9	1	100	0.50	0.33	0.31	6.13
27	DiGT5'CY	✓	54.3	10	9	2	90	0.62	0.49	0.41	4.13
31	TriCAC5'CR	-	-	-	-	-	-	-	-	-	-
34	TriCAG3'RC	-	-	-	-	-	-	-	-	-	-
35	TriCAG3'YC	-	-	-	-	-	-	-	-	-	-
36	TriCAG5'CR	✓	59.0	6	5	1	83.3	0.66	0.32	0.00	1.95
39	TriGTG3'RC	✓	57.9	6	6	0	100	0.41	0.21	0.09	4.23
40	TriGTG3'YC	✓	57.9	6	6	0	100	0.50	0.22	0.11	4.05
42	TriGTG5'CY	✓	57.7	4	3	1	75	0.60	0.14	0.02	1.63
44	TriTGT3'YC	✓	46.6	5	5	0	100	0.08	0.13	0.00	4.80
45	TriTGT5'CR	✓	46.4	5	5	0	100	0.81	0.34	0.00	1.83
46	TriTGT5'CY	✓	46.8	4	4	1	100	0.48	0.16	0.00	2.48
47	TriTGT5'CY	✓	46.4	6	6	0	100	0.39	0.19	0.07	4.70
48	TriAAT 3'RC	-	-	-	-	-	-	-	-	-	-
52	TriATT3'RC	-	-	-	-	-	-	-	-	-	-
53	TriATC3'RC	✓	44.5	5	5	0	100	0.28	0.15	0.00	4.23
54	TriATG3'RC	-	-	-	-	-	-	-	-	-	-
55	TriACA3'RC	-	-	-	-	-	-	-	-	-	-
72	TriTGA3'RC	-	-	-	-	-	-	-	-	-	-
74	TriTGA3'RC	-	-	-	-	-	-	-	-	-	-
79	TriCAC3'RC	✓	58.1	8	7	1	87.5	0.33	0.22	0.02	5.60
83	TriCTG3'RC	✓	58.8	8	5	2	62.5	0.39	0.17	0.00	3.75
86	TriCGA3'RC	-	-	-	-	-	-	-	-	-	-
87	TriCGT3'RC	-	-	-	-	-	-	-	-	-	-
90	TriGAA3'RC	-	-	-	-	-	-	-	-	-	-
91	TriGAT3'RC	-	-	-	-	-	-	-	-	-	-
93	TriGAG3'RC	✓	57.3	5	4	1	80	0.12	0.11	0.00	3.75
96	TriGTC3'RC	-	-	-	-	-	-	-	-	-	-
98	TriGTC3'RC	✓	57.3	7	7	0	100	0.50	0.30	0.17	4.15
Total	-		-	116	108	10	-	-	-	-	-
Mean	-		-	6.4	6	-	93.24	0.44	0.24	0.12	3.78

AP, Amplification product; T (°C), annealing temperature; TNB, total number of bands; NPB, number of polymorphic bands; NMB, number of monomorphic bands; PPBN (%), percentage of polymorphic band number; PIC, polymorphic information content; EMR, effective multiple relation; MI, markers index; RP, primer resolution power; ✓, with amplification; -, without amplification.

Table 3. Summary of genetic diversity parameters found by evaluating three *Manihot* populations in Angola, based on 18 ISSR primers.

Population	Na	Ne	H	I	No. of polymorphic loci	% of polymorphic (P)
Cuanza Norte	1.61 (±0.50)	1.41 (±0.39)	0.24 (±0.21)	0.35 (±0.30)	66	61.11
Malanje	1.92 (±0.26)	1.59 (±0.32)	0.34 (±0.16)	0.50 (±0.21)	100	92.59
Uige	1.48 (±0.50)	1.37 (±0.42)	0.20 (±0.22)	0.29 (±0.31)	52	48.15

Na, Number of observed alleles; Ne, number of effective alleles; H, Nei genetic diversity; I, Shannon diversity index; ± SD, standard deviation ().

Table 4. Genetic parameters of *Manihot esculenta* Crantz population.

Parameter	T_H	H_S	G_{ST}	Nm
Mean	0.3113	0.587	0.1690	2.4585
Standard deviation	0.0219	0.0183	-	-

TH, Total heterozygosity; HS, mean genetic diversity within populations; G_{ST}, genetic diversity between populations; Nm, number of immigrants ().

Table 5. Molecular analysis of variance (ANOVA) applied to the *Manhiot esculenta* populations assessed based on 18 IRRS markers.

Variation source	DF*	SS*	CV*	VT %*	P* Value
Between populations	2	59.15	1.56	10.41	
Within populations	37	498.13	13.46	89.59	<0.000
Total	39	557.28	15.02	-	

DF, Degree of Freedom; SS, sum of squares; CV, coefficient of variance; TV, total variance; P are the likelihood of having a coefficient of variance higher than the values randomly observed. The probabilities were calculated through 1023 random permutations. F_{ST} = 0.10409.

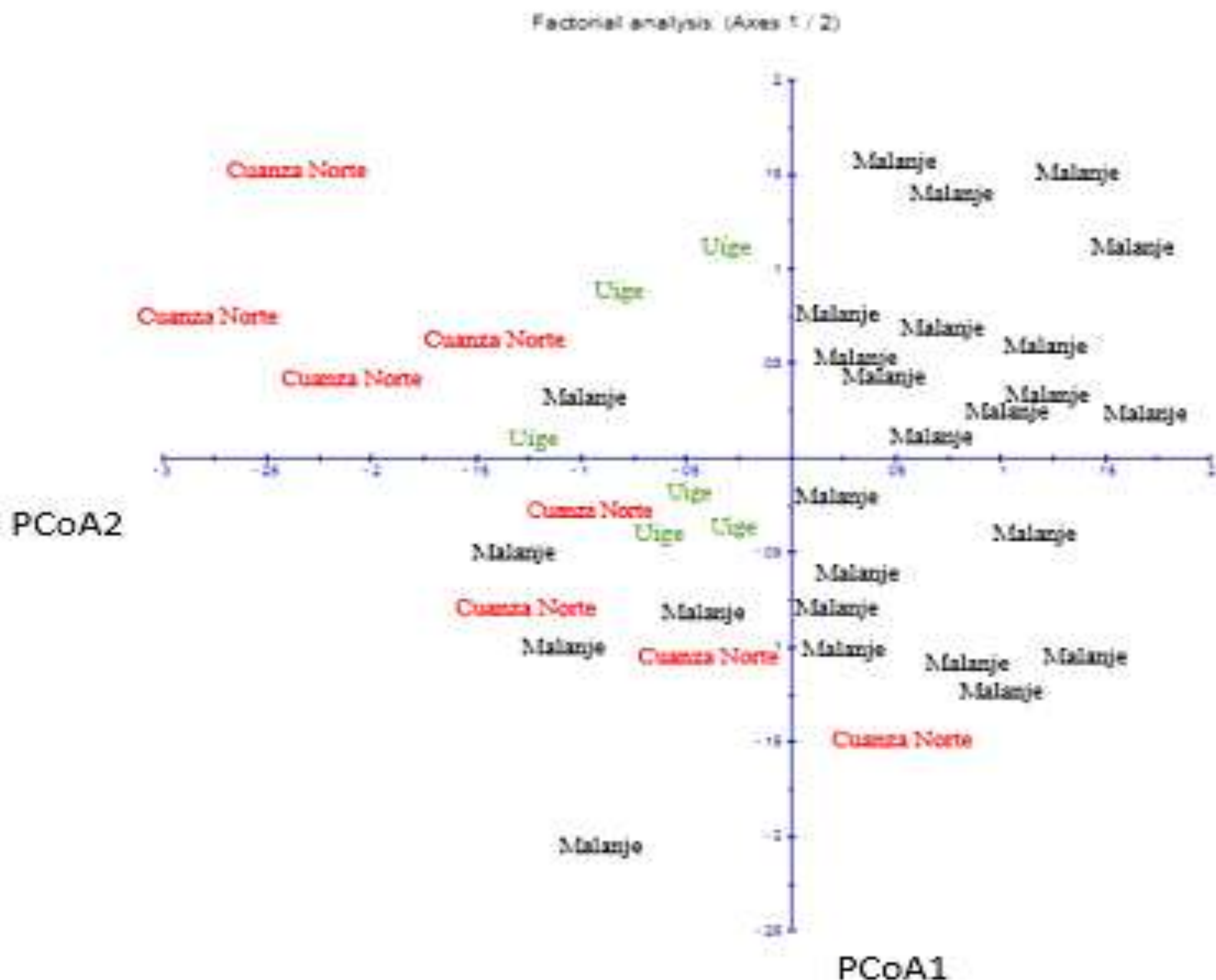

Figure 2. Scatter plot of the three assessed cassava populations.

indicated high genetic variability. Tiago et al. (2015) found 23 polymorphic bands (67.65%) by selecting 34 cassava ISSR primers presenting variation ranging from 1 to 10 and mean band 5.75 bands per primer. Turyagyenda et al. (2012) found 154 polymorphic bands by selecting 26 SSR primers for cassava grown in Uganda, which presented variation ranging from 3 to 11, and mean variation of 5.92 bands per primer. Hu et al. (2010) also observed high polymorphism and relative difference between populations in a study about the genetic variation among 13 *Rheum tanguticum* populations in China with 13 SSR markers by associating genetic distance with geographic distances. Costa (2010) assessed the molecular techniques applied to genus *Hypericum* and found high genetic polymorphism based on SSR markers.

The polymorphic information content represents the likelihood of finding the marker in its two states: absence and presence (Roldan-Ruiz et al., 2000). According to Xie et al. (2010), the PIC value of little informative markers ranges from 0 to 25 in little informative markers, from 0.25 to 0.5 in moderately informative markers and it is above 0.5 in markers presenting highly informative content. This value helps ranking the primers based on polymorphism-detection efficiency, which worked as a parameter to select the primers (Costa et al., 2015). Thus, as for the present study, it is possible considering that primer efficiency in indicating polymorphism between genotypes was moderate.

Similar results to those recorded in the current study were observed by Gonçalves et al. (2017), who found moderate PIC value (0.48) in sweet cassava populations by using ISSR markers. Kawuki et al. (2009) observed PIC values between 0.358 and 0.759 and mean value 0.571 by using ISSR markers in cassava germplasm from Africa, Asia and America. In a study about native *Prunus armeniaca* L. (Rosaceae) populations in Northeastern China, Li et al. (2013) found PIC values ranging from 0.15 to 0.27, and mean value 0.21 by using ISSR markers; primers were considered little informative. Costa et al. (2015) assessed natural *mangaba* populations (*Hancornia speciosa* Gomes) by using ISSR markers and found PIC values ranging from 0.26 to 0.44; primers were considered moderately informative.

Polymorphic information content (PIC) is taken into consideration and multiplied by the index that takes into account the effective multiple relations to calculate the marker index (MI). Primers 26 and 27 recorded the highest MI values. Although primer 27 recorded the highest MI value, it was not selected in the present study through the PIC values of markers. According to Tatikonda et al. (2009), there is no ideal specific MI value; thus, it is possible analyzing results recorded for this index by comparing the values of each primer used in the study, the best indices will always be the ones presenting the highest values.

Resolution power (RP) is a parameter that indicates the discriminating power of the marker (Tatikonda et al., 2009). Just as MI, there is no maximum RP value, since it concerns a sum of values. It was observed that primers presenting the best RP values also showed the largest number of fragments. It happens because the formula used to calculate these indices take into account the sum of I_b values. Thus, primers capable of amplifying a large number of bands tend to present higher RP values than the ones amplifying a smaller number of bands.

Primer 26 also presented high PIC and MI values and this outcome indicates that this marker is quite informative for cassava. It stood out for its high PIC, MI and PR values. The highest MI and RP values were observed in primers 26 and 27, which were the most informative ones. Thus, the discrimination of all accessions can be conducted by the smaller number of primers through the adoption of the most informative primers: 26 and 27. Reduction in the number of primers not only leads to reduction in the time adopted for the analysis, but also to cost reduction (Varshney et al., 2007). According to Smith and Pham (1996), genetic diversity estimates based on dominant markers are sometimes lower than estimates made for co-dominant markers. However, Ge and Sun (2001) state that the general standard lies on detecting higher genetic-diversity levels through dominant markers in comparison to co-dominant markers.

Results about the number of observed alleles found in the present study evidenced that values ranged from 1.48 to 1.92. Agre et al. (2017) observed Na values from 0.23 to 1.0 and mean value 0.66 by using ISSR markers in cassava grown in the Republic of Benin. The Shannon index (I) was considered an intermediate value; according to Giustina et al. (2014), Shannon indices (I) vary from 0 to 1, wherein 0 means null gene diversity and 1 is the maximum gene diversity. Silva et al. (2016) assessed the genetic diversity estimated through microsatellite markers in commercial Cupuaçu crops and observed lower Nei diversity values (0.11) and Shannon index (I) (0.17) in comparison to values recorded in the current study. Nei genetic diversity index (H) values ranged from 0.20 to 0.24. Pádua (2011) assessed a *Eremanthus erythropappus* population by using ISSR markers and observed genetic diversity indices ranging from 0.26 to 0.38.

Genetic variability results showed that the highest genetic production was observed within populations (83.10%) and this result complied with Hamrick et al. (1991), who stated that there is more intra-specific diversity than inter-specific diversity due to factors affecting the geographic distribution. Genetic diversity, or heterozygosity, is the most used factor to estimate genetic variety since it is less sensitive to variations in sample size. Therefore, sample size becomes the most important parameter when it is compared to other parameters such as the percentage of polymorphic *loci* and the mean number of alleles per *locus,* besides its

easy genetic interpretation (Brown and Weir, 1983). The result of total genetic diversity (HT) found through the Nei Index (1973) reached estimated mean 0.3113, and this outcome points towards high heterozygosity in *Manihot* populations. These values were considered high and they indicated that the variability between and within populations would contribute 31.13 and 68.87%, respectively. This value was close to that found by Teixeira et al. (2012), who worked with *Campomanesia* species populations by using ISSR markers. These authors observed mean value 0.365, and it confirmed that the populations presented genetic variability.

According to Loveless and Hamrick (1984), if the gene flow is restricted, populations would show high divergence. The value observed in the present study (2.4585) showed limited gene flow among the three assessed populations. This value was not enough to counterpoint the effects of genetic drift. This value should be higher than four migrants per generation (Nm> 4), so that the homogenizing effect of the gene flow overlaps the genetic drift (Slatkin and Barton, 1989; Hart and Clark, 1997). Wright (1931) states that gene flow values lower than 1 indicate genetic isolation. According to Wright (1951), gene flow values higher than 1 are enough to stop random allele losses within populations (drift effects). Estimates based on data from dominant molecular values in three cassava populations and calculated through ANOVA evidenced 89.59% genetic divergence, on average, within populations. Genetic divergence results in the present study showed 10.41% distribution between populations. These data are lower than the ones reported by Brandão et al. (2011), but they comply with the numbers recorded by Fernandes (2008), who assessed *Caryocar brasiliense* populations.

The G_{ST} value showed greater variation within populations than between populations and this outcome corroborated the ANOVA results. The evolutionary geographic and historical distribution plays a relevant role in genetic variation distribution between and within populations (Hamrick et al., 1992). However, this variation may depend on the presence or absence of certain alleles in geographic regions. The expectation is to observe that the greater the geographic distribution the greater the diversity of this species (Bozza, 2009).

The analysis of the mean coordinates (PCoA) was calculated based on coefficients of dissimilarity and graphically presented in Figure 2. The coordinates were calculated for the two first axes with negative Eigen values. The two axes represented 51% total variation; the first axis (PCoA1) represented 28.54% and the second one (PCoA2), 22.46%. Individuals grouped by geographic location indicated that, although they originated from different locations, they belonged to the three populations, except for the first coordinate, which was represented by individuals belonging to the Malanje and Cuanza Norte populations and for the second coordinate, with individuals belonging to Malanje, Uige and Cuanza Norte populations. A result similar to that recorded in the present study was found by Turyagyenda et al. (2012), who observed PCoA1 and PCoA2 values ranging from 23.56 to 20.06%, respectively, and 43.62% total variation by using SSR markers in cassava grown in Uganda.

The analysis of main components depicted the relations between different geographic areas, besides highlighting that the largest number of individuals corresponded to variety Malanje. This analysis showed wide dispersion of individuals and evidenced wide genetic variability distribution. These results also showed that most of the assessed individuals shared an important part of their genetic information. It was possible observing that there were some individuals in the three populations that got far from each other, and this outcome evidenced that these individuals are the ones that mostly contributed to the genetic variability.

The genetic structure in *M. esculenta* was characterized through Bayesian analysis, which took into account the separation of the total number of individuals in groups (clusters) by attributing a K number of populations to them and by assuming that these individuals present Hardy-Weinberg equilibrium (Figure 3). The ΔK of each K value was calculated (Evanno et al., 2005) and it allowed an easy interpretation of K, which is the most likely value to represent the number of groups in the data matrix. According to Vigouroux et al. (2008), if a certain variety presents a value higher than the arbitrary limit (80%) of its genome in a group, then, it belongs to the referred group.

Knowledge about the genetic structure of the populations is essential for the efficient use of genetic resources, as well as for better understating their evolutionary history (Venkovsky et al., 2007; Clement et al., 2010). Based on Figure 4, when K=1, genotypes from Cuanza Norte, Malanje and Uíge were formed by three great ancestor groups, basically, the green, blue and red ones. When K=2, it was possible observing that the population from Malanje basically remained in group blue, which was formed by eight genotypes. There was allelic richness when K=3, although the marker was dominant; therefore, there was allele sharing between one or the other populations. This group was composed of genotypes from Malanje and Uíge and presented the formation of two ancestor groups, basically the blue and the red ones.

These results suggested genetically defined groups that corresponded to the pre-defined regional groups. Therefore, farmers select cassava varieties for different purposes, and this outcome also highlights material exchange or gene flow between locations, mainly in Malanje, which was the most heterogeneous community within the group. Muhlen et al. (2012) used microsatellite markers and showed groupings by separating sweet from bitter cassava. Their results were similar to those recorded by Emperaire et al. (2003) and Elias et al. (2004), who detected geographic structuration in cassava varieties.

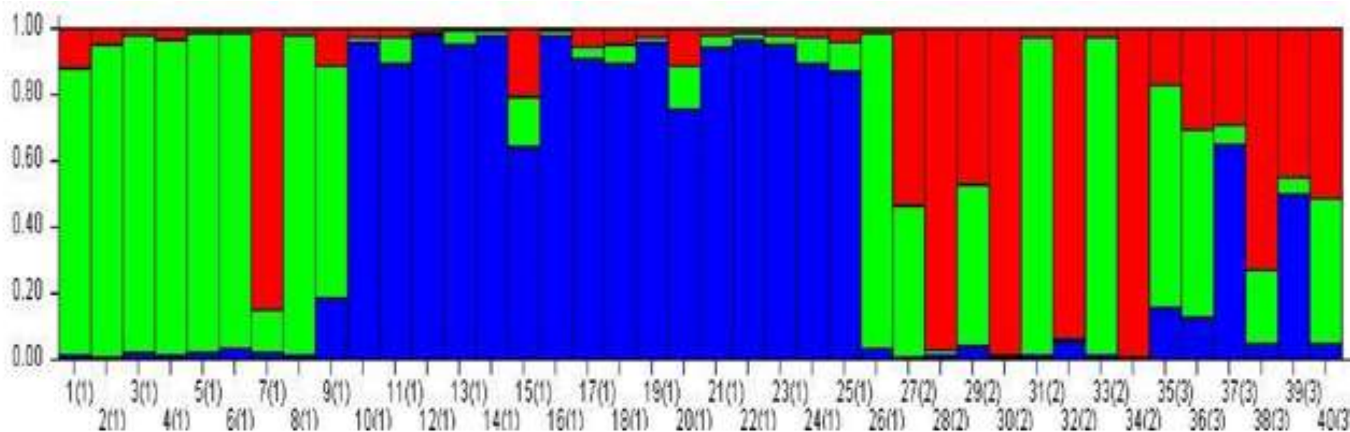

Figure 3. ΔK value of the possible grouping of 40 Angolan cassava varieties deriving from 10 structure analysis simulations.

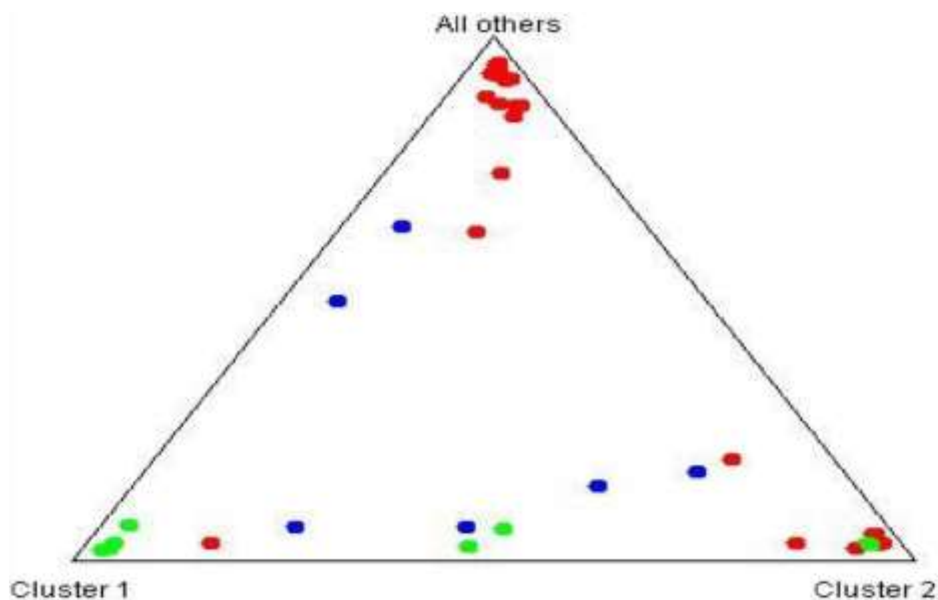

Figure 4. Representation of the number of K groups for 40 cassava individuals in the three assessed populations based on ISSR molecular data calculated in the structure software (Three groups, K=3).

This analysis allowed predicting the structure of the populations and the ancestry of the individuals; thus, it contributed to the development of new varieties. The greatest concentration of genetic variability in Malanje and Cuanza Norte was likely related to the exchange and introduction of material in the crops. The observed variability can be explained by the adaptation of the species to different environments. There was low gene flow (Table 4) and wide genetic variability distribution within populations (Table 5); thus, it was possible confirming the genetic structuration between the assessed populations. According to Bozza (2009), the wider the geographic distribution, the greater the diversity of the species.

Conclusion

Molecular featuring evidenced genetic diversity within each assessed population. The structure division in the three main groups showed genetic information sharing and the consequent insertions of individuals belonging to the Malanje population in the Cuanza Norte group. The PCoA analyses led to the evolutionary connection of these areas. The highest concentration of genetic variability in the Malanje and Cuanza Norte regions were

possibly related to the exchange and introduction of material in the crops.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank all the farmers involved in this study and the partner institutions for the resources that made this work possible. The National Institute of Management of Scholarships (INAGBE) awarded a doctoral scholarship to Sandra Domingos João Afonso.

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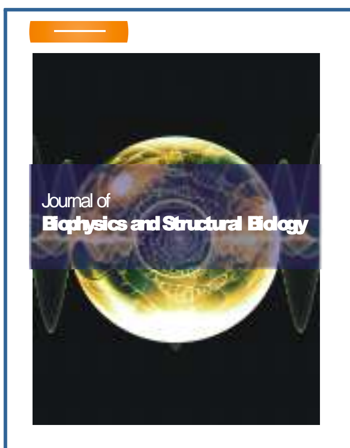

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